# Antioxidant properties of ticlopidine on human low density lipoprotein oxidation

Domenico Lapenna*, Sergio de Gioia, Giuliano Ciofani, Cristiana Bruno, Ettore Porreca, Sante D. Pierdomenico, Franco Cuccurullo

*Dipartimento di Medicina e Scienze dell'Invecchiamento, Università degli Studi 'G. d'Annunzio', Facoltà di Medicina e Chirurgia, Chieti, Italy*

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**Abstract** **We found that ticlopidine, at therapeutically relevant concentrations (2.5–10 μM), but not aspirin nor salicylate, significantly counteracted copper-driven human LDL oxidation. Ticlopidine, at 5 and 10 μM, was also antioxidant on peroxyl radical-induced LDL oxidation; yet it was ineffectual on thiol and ascorbate oxidation mediated by peroxyl radicals themselves, suggesting that drug antioxidant capacity is somehow related to the lipoprotein nature of the oxidizable substrate, but not to radical scavenging. The drug could not indeed react with the stable free radical 1,1-diphenyl-2-pycrylhydrazyl, nor had apparent metal complexing-inactivating activity. Thus, ticlopidine has antioxidant effects on LDL oxidation, which, together with its anti-platelet activity, could confer peculiar antiatherogenic properties to the drug in vivo.**



*Key words:* Ticlopidine; Low density lipoprotein oxidation; Antioxidant; Atherosclerosis

## 1. Introduction

In recent years evidence has accumulated indicating that oxidized low-density lipoproteins (LDL) play a central role in atherogenic processes and that LDL oxidation can be prevented by antioxidant compounds [1–3].

Some antioxidants seem to exert specific effects on LDL or other lipid system oxidation as a consequence of their lipophilicity, resulting in a less lipid propensity to undergo radical-driven oxidant damage [4–9]. Ticlopidine, a widely used anti-platelet agent with a proven clinical efficiency [10–12], is a thienopyridine characterized by lipophilic properties (Fig. 1); the drug is indeed strictly associated with the lipid moiety of human lipoproteins, such as LDL [13]. Moreover, the presence of sulfur in the chemical structure of ticlopidine (Fig. 1) might also suggest some scavenging activity of the drug against radical species involved in lipid and LDL oxidation, such as peroxyl radicals, as reported by Pryor and associates for probucol [14]. Thus, some antioxidant activity of ticlopidine on LDL oxidation may be hypothesized.

The present study was designed to investigate possible antioxidant properties of ticlopidine on human LDL oxidation. For comparison, the effects of another well known anti-platelet drug, namely aspirin, and of its main metabolite salicylate, were also studied. The results show that ticlopidine only is capable of inhibiting LDL oxidation in specific model systems, this effect being evident at therapeutically achievable concentrations.

## 2. Materials and methods

### 2.1. LDL isolation and oxidation

Reagents were from Sigma Aldrich, Milano, Italy, except for 2,2′-azobis(amidinopropane) dihydrochloride (AAPH), which was from Polysciences, Warrington, USA. LDL were isolated from EDTA plasma of healthy subjects (age 26–52 years) by density gradient ultracentrifugation at 4°C essentially as reported by Frei and Gaziano [15]. After puncture aspiration of the LDL band, samples were extensively dialyzed against phosphate buffered saline (PBS), pH 7.4, at 4°C.

Drugs were used at therapeutically relevant final concentrations, namely 2.5, 5 and 10 μM for ticlopidine, and up to 100 and 200 μM for aspirin and sodium salicylate, respectively [16–18].

Drug capability to inhibit copper-mediated LDL oxidation was evaluated in quartz cuvettes through continuous spectrophotometric monitoring of absorbance increase at 234 nm, reflecting conjugated dienes (CD) formation during peroxidative processes [19,20]. In this regard, a kinetic study of the duration of the lag phase, of the rate of CD production and of the maximal yield of CD generation was specifically performed [19,20]. Oxidation of LDL (0.1 mg LDL protein/ml) was induced by 5 μM $CuSO_4$ in PBS, pH 7.4, with and without the drugs, using appropriate LDL- and drug-containing blanks. Results were calculated as nmol CD/mg LDL protein, using a molar extinction coefficient of 29 500 at 234 nm [19,20]. In some experiments, drug effects on copper-catalysed LDL oxidation were assessed measuring aldehydic by-products of lipoperoxidation through a thiobarbituric acid (TBA) test, after 2 h incubation at 37°C of LDL (0.1 mg LDL protein/ml) with 5 μM $CuSO_4$ in PBS, pH 7.4. TBA reactive substances (TBARS) were assayed basically according to Ohkawa et al. [21], with some modifications [22]. Briefly, a suitable aliquot of the LDL sample was added to a reaction mixture containing 0.25 mM EDTA, 20 μM butylated hydroxytoluene in absolute ethanol, 0.1 ml of 8.1% sodium dodecyl sulfate, 1.3 ml of 20% acetic acid, pH 3.5, and 1.3 ml aqueous solution of 0.6% TBA, followed by 30 min heating at 95°C. After cooling and centrifugation, the chromogen was read at 532 against an appropriate blank. Results were calculated as nmol TBARS/mg LDL protein, using a molar extinction coefficient of 154 000. No pharmacological interference was observed in the TBA-test.

When a drug was found to afford protection against copper-mediated LDL oxidation, further investigations were specifically performed to examine the mechanisms involved in drug antioxidant protection (see below).

In other specific experiments, therefore, the oxidation of LDL (0.1 mg LDL protein/ml) was induced in a metal-independent fashion through the peroxyl radical-generating azo-initiator AAPH (4 mM) in the presence of 0.1 mM diethylenetriaminepentaacetic acid (DTPA) [15], allowing 3 h incubation at 37°C in PBS, pH 7.4. LDL oxidation was assessed through the TBA-test, as reported above.

LDL-protein was measured by the method of Lowry et al. [23].

### 2.2. Drug radical scavenging activity

Drug capability to inhibit AAPH-mediated, peroxyl radical-induced oxidation of different non-lipid substrates, namely the thiol compound 5-thio-2-nitrobenzoic acid (TNB) and ascorbic acid, was investigated. TNB was prepared through reduction of 5,5′-dithiobis(2-nitrobenzoic acid) with 2-mercaptoethanol as reported by Thomas et al. [24], and

*Corresponding author. Cattedra di Patologia Medica, c/o Policlinico di Colle dell'Ara, Via dei Vestini, 66100 Chieti, Italy.
Fax: (39) (871) 551615.


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its concentrations were calculated using a molar extinction coefficient of 13 600 at 412 nm [24]. Reaction mixtures contained 37 μM TNB, 0.1 mM DTPA and 4 mM AAPH, with or without various drug concentrations, in PBS, pH 7.4. Incubation was for 3 h at 37°C. Absorbance values at 412 nm ($A_{412}$) were then recorded spectrophotometrically against appropriate blanks to detect specific drug effects. For ascorbate oxidation, reaction mixtures contained 55 μM ascorbic acid and 8 mM AAPH, with and without ticlopidine, in pyrogen-free physiological saline, where the vitamin is quite stable and virtually does not autooxidize. After 90 min incubation at 37°C, ascorbate-related absorbance values at 265 nm were recorded spectrophotometrically against appropriate blanks. Results were calculated as nmol ascorbic acid oxidized/ml/min, using a molar extinction coefficient of 15 000.

We also evaluated drug capacity to interact with and scavenge the stable free radical 1,1-diphenyl-2-pycrylhydrazyl (DPH), inducing 1,1-diphenyl-2-picrylhydrazine formation and DPH bleaching [25,26]. It should be noted that reaction with DPH is typical of those substances acting as antioxidants by virtue of their radical scavenging properties [25,26]. The reaction system contained 90 μM DPH (previously dissolved in ethanol) in PBS, pH 7.4, with or without various drug concentrations. In some experiments, the reaction was carried out directly in organic solvents, such as ethanol or chloroform. After 5 min incubation at 25°C, DPH-related absorbance values at 517 ($A_{517}$) were recorded spectrophotometrically against appropriate drug-containing blanks.

### 2.3. Drug-copper interaction

Possible drug interactions with copper were investigated through an ultraviolet spectral study (from 270 to 200 nm) of ticlopidine with either copper(II) as $CuSO_4$ in PBS, or copper(I) as CuCl in argon-purged acetonitrile. To further study drug-copper interaction, fluorescence-quenching experiments were specifically carried out for ticlopidine, which shows an intrinsic fluorescence. Indeed, the relative fluorescence of a substance can be quenched by catalytic transition metals, as a result of formation of a complex between such a substance and metals themselves [22,27]. Excitation and emission wavelengths of ticlopidine were determined to be 321 and 435 nm; the drug was used at a final concentration of 10 μM, while the concentrations of $CuSO_4$ (which gave no interference at the afore-mentioned wavelengths) ranged from 1.0 to 100 μM. Finally, drug effects on copper-induced ascorbic acid oxidation were investigated [28]. Reaction mixtures contained 37 μM ascorbic acid and 5 μM $CuSO_4$, with and without ticlopidine, in pyrogen-free physiological saline. After 15 min incubation at 37°C, ascorbate-related absorbance values at 265 nm were recorded spectrophotometrically against appropriate drug-containing blanks. Results were calculated as nmol ascorbic acid oxidized/ml/min.

### 2.4. Statistics

Data were calculated as means ± S.D. of 7 different experiments, unless otherwise indicated. Specific drug effects were evaluated by the one-way analysis of variance (ANOVA) plus Student-Newman-Keuls test [29]. $P < 0.05$ was regarded as statistically significant.

## 3. Results

### 3.1. Drug effects on LDL oxidation

As shown in Table 1 and in Fig. 2, ticlopidine significantly influenced the kinetics of copper-driven LDL oxidation. Indeed, lag time was prolonged in a dose-dependent fashion by ticlopidine, resulting about 1.5-, 2.3- and 4-fold longer than that of control at 2.5, 5 and 10 μM drug concentrations, respectively (Table 1). Moreover, oxidation rate and maximal yield of CD production were decreased by ticlopidine, a significant effect being evident, however, beginning from 5 μM drug concentration (Table 1). In the same experimental conditions, either aspirin or salicylate were ineffective (data not shown). When copper-catalyzed LDL oxidation was evaluated through TBARS assay, 67 ± 9.5 nmol TBARS/mg LDL protein were generated in control experiments; TBARS were instead 58.5 ± 11 and 33 ± 7.5 nmol/mg LDL protein in the presence of 2.5 and 5 μM ticlopidine ($P < 0.05$; $n = 6$), and they were virtually undetectable with 10 μM ticlopidine, further indicating an effective antioxidant activity of the drug.

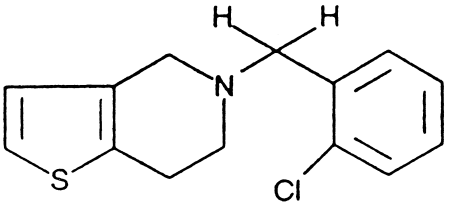


Fig. 1. Chemical structure of ticlopidine.

Moreover, ticlopidine, at 5 and 10 μM concentrations, significantly counteracted AAPH-mediated, peroxyl radical-induced LDL oxidation, which resulted in 38.2 ± 4.7 nmol TBARS/mg LDL protein in control experiments, as compared to 34.4 ± 4.1, 28.7 ± 2.8 and 26 ± 3.1 nmol TBARS/mg LDL protein with 2.5, 5 and 10 μM drug concentrations, respectively (5 and 10 μM ticlopidine vs. control, $P < 0.05$; $n = 5$).

### 3.2. Drug radical scavenging activity

To investigate whether the antioxidant activity of ticlopidine on peroxyl radical- and copper-induced LDL oxidation could be related to its direct radical scavenging properties or to the lipoprotein nature of the oxidizable substrate, we assessed drug effects on AAPH-mediated, peroxyl radical-induced oxidation of the non-lipid compounds TNB and ascorbic acid. Ticlopidine was incapable of inhibiting such oxidations; in fact, incubation with AAPH resulted in a marked decrement of TNB-related $A_{412}$ values (from 0.502 ± 0.032 to 0.037 ± 0.006, $n = 5$), and ticlopidine, even at

Table 1
Effect of ticlopidine on the kinetics of copper-mediated human LDL oxidation

| | Lag time (min) |
|---|---|
| Control | 48.7 ± 6.2 |
| Control plus ticlopidine: | |
| 2.5 μM | 77.15 ± 7.4* |
| 5 μM | 112.4 ± 17.3*,** |
| 10 μM | 200.8 ± 30*,** |
| | **Oxidation rate (nmol CD/min/mg LDL protein)** |
| Control | 2.8 ± 2.4 |
| Control plus ticlopidine: | |
| 2.5 μM | 11.8 ± 1.9 |
| 5 μM | 9.7 ± 1.7*,*** |
| 10 μM | 8.35 ± 1.4*,*** |
| | **Maximal CD production (nmol CD/mg LDL protein)** |
| Control | 402.4 ± 32.5 |
| Control plus ticlopidine: | |
| 2.5 μM | 385.6 ± 30 |
| 5 μM | 347.3 ± 31.5*,*** |
| 10 μM | 333.7 ± 25*,*** |

Means ± S.D. of 7 different experiments. CD: conjugated dienes. *$P < 0.05$ vs. control; **$P < 0.05$ vs. preceding values; ***$P < 0.05$ vs. 2.5 μM ticlopidine (ANOVA plus Student-Newman-Keuls test). See Sections 2 and 3 for further methodological explanations.

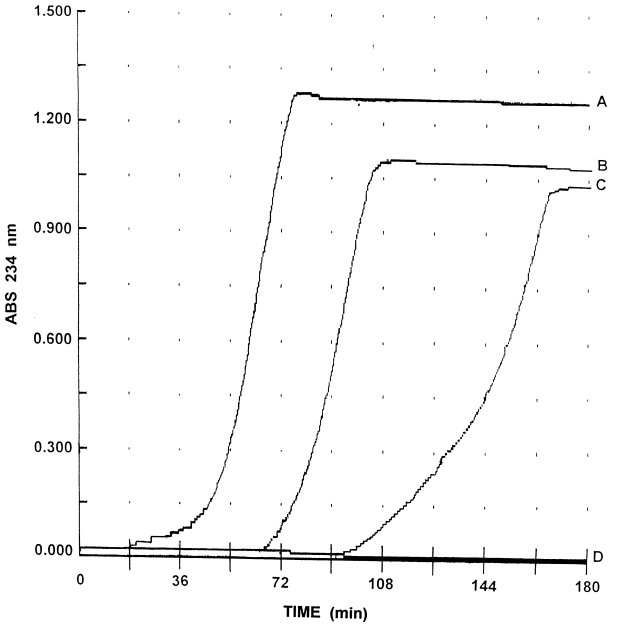


Fig. 2. Antioxidant activity of ticlopidine on human LDL oxidation. Incubation of LDL was at 37°C in PBS, pH 7.4, with 5 μM $CuSO_4$ in the absence (trace A, control) or presence of 2.5, 5 and 10 μM ticlopidine (traces B, C and D, respectively). Conjugated diene formation during oxidation was followed spectrophotometrically in quartz cuvettes as an increase of absorbance at 234 nm (ABS 234 nm). Figure represents results from a typical kinetic experiment (see Sections 2 and 3 for further explanation).

10 μM, was ineffective ($0.035 \pm 0.005$ $A_{412}$, $P = \text{NS}$; $n = 5$). Moreover, $0.42 \pm 0.027$ nmol/ml/min ascorbic acid were oxidized by AAPH-generated peroxyl radicals, not different from the values detected with 10 μM ticlopidine ($0.43 \pm 0.023$ nmol/ml/min, $P = \text{NS}$; $n = 5$). In line with these experiments, ticlopidine could not interact with the stable free radical DPH, further suggesting no direct radical scavenging activity of the drug [25,26]. DPH-related $A_{517}$ values were indeed $0.603 \pm 0.030$ in control experiments, not significantly different from those observed with 10 μM ticlopidine ($0.608 \pm 0.035$, $P = \text{NS}$; $n = 5$). Similarly, the drug was ineffective when the reaction was carried out in organic solvents (not shown).

Overall, these data indicate that the specific antioxidant effects of ticlopidine are apparently related to the (lipid) nature of the oxidizable substrate, namely LDL, and not to a direct radical scavenging activity of the drug.

### 3.3. Drug-copper interaction

Ticlopidine had no apparent copper-binding effect, as judged by both spectral study and lack of quenching of the intrinsic drug fluorescence by copper (not shown). Furthermore, the drug did not influence copper-driven ascorbate oxidation, suggesting that the metal redox potential and catalytic activity is substantially unaffected by the drug [28]. Indeed, $0.78 \pm 0.1$ and $0.8 \pm 0.08$ nmol/ml/min of ascorbic acid were oxidized by copper with and without 10 μM ticlopidine, respectively ($P = \text{NS}$; $n = 5$).

## 4. Discussion

The present study shows that ticlopidine, but not aspirin or salicylate, can inhibit human LDL oxidation. Three mechanisms should be basically considered in the pharmacological antagonism of copper-mediated LDL oxidation: (i) metal complexation-inactivation; (ii) scavenging of radical species involved in LDL oxidation, such as peroxyl, alkoxyl or lipid radicals; (iii) stabilization of the lipid moiety of LDL, conceivably via chemical interactions between drug hydrophobic groups and polyunsaturated residues of LDL phospholipids. Our data indicate no specific antioxidant effects of ticlopidine as a result of copper complexation-inactivation. Even though the antioxidant activity of ticlopidine on AAPH-mediated LDL oxidation may suggest drug scavenging properties against peroxyl radicals, other experimental data herewith reported do not point to a similar mechanism. Indeed, ticlopidine is ineffective on the oxidation of the non-lipid compounds TNB and ascorbate mediated by AAPH, and it does not interact with the stable free radical DPH. Regarding AAPH, it seems to form an equilibrium between the lipid and aqueous environment, with peroxyl radicals being generated in both phases [30]; thus, it is possible that a lipophilic drug capable of stabilizing LDL phospholipids could afford some protection against AAPH-mediated LDL oxidation. It cannot be totally excluded, however, that ticlopidine might specifically interact with some (lipid) radicals generated during copper-catalysed oxidative processes directly within the hydrophobic core of LDL. Given the lipophilic nature of ticlopidine and its capacity to bind to lipids in LDL [13], the antioxidant properties of the drug may be directly related to a stabilization of the phospholipid moiety of LDL with decreased lipid propensity to undergo radical-driven oxidant damage. Consistent with this, it has recently been reported that the 3-hydroxy-3-methylglutaryl coenzyme A inhibitor fluvastatin can inhibit both copper and AAPH-mediated LDL oxidation apparently as a result of drug binding to LDL phospholipids with prevention of radical diffusion into the lipoprotein core [31]. Moreover, lipophilic drugs, such as phenothiazines, calcium antagonists, beta-blockers and tamoxifen, are known to exert inhibitory effects on LDL oxidation [4,6,8,9]. Indeed, the role of the physical state of the lipid phase in the susceptibility of lipids to peroxidative processes has been pointed out [32,33].

Plasma peak concentrations of ticlopidine are up to 7 μM after drug administration of 500 mg/day in young volunteers, and ticlopidine plasma levels are in elderly patients twice those found in young men [16,17]; however, far higher concentrations may be expected in the lipoprotein compartment, where the drug is bound [13]. Thus, the antioxidant activity of ticlopidine on LDL oxidation is evident at therapeutic concentrations and appears feasible in the clinical setting. A lipophilic antioxidant strictly associated with LDL may delay LDL oxidation especially in the vascular wall and thus the development of atherosclerosis. Evidence for the relevance of extending the lag time for LDL oxidation is provided by clinical studies indicating an inverse relationship between the duration of such lag time and the severity of atherosclerosis [34]. The efficacy of ticlopidine in the prevention of atherosclerosis-related cardiovascular diseases has been recognized [10–12], and the drug has been reported to be even superior to aspirin in specific studies [10–12]. The previously unrecognized antioxidant effects of ticlopidine on LDL oxidation, together with its well-known anti-platelet activity, could confer peculiar antiatherogenic properties to the drug in vivo.

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